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TITLE: p53 Regulation of Uridine Phosphorylase Activity, Pyrimidine Salvage Pathway and Their Effects on Breast Cancer Therapy

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13. ABSTRACT *(Maximum 200 Words)*

This research project has been focused on the elucidation of the mechanism affecting the therapeutic efficacy of fluoropyrimidines to improve the clinical outcome of cancer patients. Based on the findings of the last scientific year that the nullification in ES cells of uridine phosphorylase (UPase) leads to a significantly increased cell resistance to fluoropyrimidines and that wild-type p53 protein down-regulates UPase expression, we have focused this year's scientific research on the following two areas: 1) Elucidation of the p53 regulation mechanism(s) of UPase expression; In this investigation, we demonstrated that wild-type p53 protein represses UPase gene expression via sequence-specific DNA binding at promoter level, and that the mutation of p53 leads to loss of this regulatory function. 2) In vivo study of UPase function in fluoropyrimidine metabolism and uridine regulation. In order to better translate the basic research understanding to the clinical service, we have extended our first year's findings in UPase knockout ES cell to in vivo study using the UPase knockout mouse model generated. The research results defined in vivo the important role of UPase in fluoropyrimidine metabolism and uridine regulation in plasma and tissue.

14. SUBJECT TERMS
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Table of Contents

Introduction

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Body

A. Scientific accomplishments

1. p53 represses UPase gene expression by sequence-specific DNA binding

We have reported a potential p53-binding element present in the UPase promoter region (1). In the current study, we extensively investigated the mechanism of p53 regulation of UPase gene expression. To observe the bioactivity of this p53-binding element, we generated promoter-luciferase constructs with or without this element and tested their promoter activity in both EMT6 and NIH 3T3 cells which both express wild-type p53 protein. As shown in Figure 1, the promoter without p53-binding element (construct -274) displays an obviously enhanced activity, compared with the promoter containing this putative p53-binding element (construct -1605 and -445). This difference was not present in p53 null murine embryo fibroblast (MEF p53 -/-) cells (data not shown). The co-transfection of the UPase promoter-luciferase constructs -1605 bp (whole length) and -445 bp with murine wild-type p53 gene resulted in a significant reduction of luciferase activity in both EMT6 and NIH/3T3 cells, but not influencing the activity of the construct -274 (Figure 1). These data indicate that the existence of this p53-binding element in the UPase promoter region negatively regulates the expression of UPase gene. Gel mobility shift assay and DNase I footprinting indicated the specific binding of this element with the p53 protein (Figure 2). The p53-dependent down-regulation of UPase gene expression was confirmed when we investigated UPase

expression in MEF p53 -/- and p53 +/+ cells using Western blot and enzyme activity assay. As showed in Figure 3, UPase protein is obviously enriched in MEF p53 -/- cells compared with that in MEF p53 +/+ cells; and the enzyme activity of UPase in MEF p53 -/- is approximately 30-fold higher than that in the MEF p53 +/+ cells. These data suggest the repressive function of p53 on UPase expression, via sequence-specific DNA binding.

To better translate this finding into a clinical relevant observation, we are currently evaluating 152 frozen breast tumor samples for p53 mutation and UPase expression and planning to establish a cause-effect correlation.

2. In vivo study on UPase role in fluoropyrimidine metabolism and homeostatic regulation of uridine in plasma and tissues.

In the first scientific year, we found that UPase activity has a great influence on the anti-proliferative activity of 5-fluoropyrimidines (Appendix, reprint). Thus, we believe that an extensive in vivo study on UPase function will provide more significant data for a better management of clinical cancer patients. To better focus the clinical relation, we stressed the in vivo role of the UPase and its p53 regulation using the UPase-nullified mouse model generated with gene targeting technology. The preliminary data have confirmed our hypothesis.

i). Generation of UPase knockout mice

Selected UPase +/- ES cells have been introduced into C57BL/6J blastocysts, which then completed their development in pseudopregnant C57BL/6J host. The chimeric offsprings identified by coat colors have been mated with C57BL/6J to generate the F1 UPase +/- mice. F2 UPase +/+ and UPase -/- littermates are produced from F1 UPase +/- males and females and used in the experiments. The genotype of mice has been defined by Southern blot analysis (Figure 4) and confirmed by detection of its products using reverse transcription polymerase chain reaction (RT-PCR) (Figure 5) and enzyme activity assay (Table I). Our data show that in the investigated major organs of UPase -/- mice the uridine phosphorolytic activity is completely abrogated. Uridine kinase (UK) is induced in spleen, liver, and kidney (30.6%, 60.9%, and 73.1% respectively), as shown in ES cells. However, no enzymatic activity changes in other organs observed. OPRTase activity was not altered (Table I).

ii). Metabolic abnormality of uridine and pyrimidine ribonucleotides in UPase knockout mice

We have hypothesized that the complete abrogation of uridine phosphorolytic activity will first affect the metabolism of uridine in plasma and tissues. Thereby we have first measured the plasma uridine level using high-performance liquid chromatography (HPLC) analysis. Our data show a 5 to 6-fold increase in the circulating uridine level in UPase -/- mice and about 2-fold elevation in UPase +/- mice, compared with UPase +/+ mice (Table II). In physiological condition, uridine degradation is completed by a series of enzymatic reactions initiated by UPase, finally forming β-alanine, an important neurotransmitter in brain and a precursor for carnosine (a substance associated with important neurological functions). After nullification of UPase activity, the uridine of plasma is regulated mainly relying on the excretion through urine or others. Thereby we have measured the uridine concentrations in urine and found a 24-fould increase in UPase -/- mice and a 4-fold increase in UPase +/- mice, compared with UPase +/+ mice (Table II). Because of the presence of an active transport mechanism in normal tissues, we have postulated that the nullification would affect the uridine levels in tissues. Therefore, uridine concentrations in brain, heart, lung, liver intestine, spleen and kidney have been analyzed by HPLC, and the preliminary data have confirmed our initial hypothesis, showing up to 20-fold increase in tissue uridine concentrations (Table II).

For pharmacokinetic analysis, we injected i.p. a tracer dose (25 μCi/mouse) of [^{3}H]uridine to UPase -/- mice. The plasma [^{3}H] uridine was separated by HPLC and determined by liquid scintillation counter (Beckman). As showed in Figure 6, we have observed a very rapid disappearance of [^{3}H] uridine from the plasma in UPase +/+ mice, with a T ½ < 2 min. On the contrary, [^{3}H] uridine T ½ is approximately 15-18 min in UPase -/- mice and a significant amount of radioactivity is still present as [^{3}H] uridine at 60 min after the administration. To trace the metabolic fate of [^{3}H] uridine, we have examined the presence of [^{3}H] uracil by HPLC analysis. At 5 min after the administration, an obvious [^{3}H] uracil peak is present in UPase +/+ mice, but not in UPase -/- mice (Figure 7), indicating the disappearance of [^{3}H] uridine in UPase +/+ mice by fast phosphorolysis.

iii). Drug resistance of UPase knockout mice

It has been our core goal to observe the response of UPase -/- mice to 5-fluouracil (5-FU) and 5'-deoxy-5-fluorouridine (5'DFUR), an intermediate metabolite of capecitabine (only oral

fluoropyrimidine approved by FDA) activation process. As showed in Figure 8, 85 mg/kg of 5-FU administered i.p. weekly represents the MTD in UPase +/+ mice causing 15-20% weight loss within 3 weeks. A higher dose level at 100 mg/kg results in a rapid death of the animals after a single dose of 5-FU. In UPase -/- mice, however, 100 mg/kg of 5-FU did not cause any toxic effect even after the administration of the 5th dose. Higher doses up to 200 mg/kg of 5-FU caused toxicity comparable to the one observed for the 85 mg/kg in UPase +/+ mice. The effect of UPase-knockout on 5'DFUR toxicity is more significant. In an experiment of 5'DFUR toxicity, we administrated 5'DFUR o.p. daily at 500 mg/kg to both UPase -/- and UPase +/+ mice (six in each treatment group, male : female = 1:1) and the mice were weighed every other day. In UPase +/+ mice, the severe toxicity was present after second dose and 3 of 6 died of the severe gastrointestine toxicity on day 3. In UPase -/- group, however, no significant toxicity was present till dose 3 and no mice death occurred till day 7 when we ended our observation because of the death in UPase +/+ group. These data prove in vivo the important role of UPase in both 5-FU and 5'DFUR activation.

B. Training accomplishments

In past year I have attended the 63rd Annual Meeting of American Association for Cancer Research (AACR) in San Francisco, 2002 and I was awarded an AACR-Aventis Scholar-in-Training Award in recognition of the promising research and meritorious submission. This meeting gave me the chance to meet many excellent scientists and pioneers in my research field. We had very constructive conversations and exchanges of our research experience and new approaches for the future, which are very helpful for my current research and future development in cancer research.

I attended several seminar series held by Yale Comprehensive Cancer Center. These seminars are usually given by excellent scientists who stand in the frontier in their research field. I have also attended seminars of experimental therapeutic program held once every two weeks. Through these seminars, I have been exposed to the new knowledge and technologies employed in cancer research, providing help with my current research and future scientific career.

Key research achievements

A. UPase is a key enzyme in fluoropyrimidines therapy and in homeostatic regulation of uridine plasma and tissue

concentrations

1. UPase plays an important role in activation of 5-FU and 5'DFUR, which has been confirmed at both cultured cell and in vivo levels.
2. UPase is a key enzyme to regulate the uridine level in both plasma and tissues.

B. p53 down-regulates the UPase expression

1. Wild-type p53 protein represses UPase gene expression via sequence-specific DNA binding at the promoter level
2. p53 mutation or abrogation abolishes its repressive action on UPase gene expression.

Reportable outcomes

1. A Scholar-in-Training Award (AACR, 2002)
2. One reprint

Conclusion

In the 2nd scientific year supported by the award, we extended and confirmed in the knockout mouse model the research results of 1st year. UPase is an important protein involved in the activation of fluoropyrimidines and uridine regulation. The abrogation of UPase in mice results in resistance to 5-FU and 5'DFUR, and the disturbance of uridine and ribonucleotide metabolism. We have also elucidated the regulatory mechanism of p53 on UPase gene expression and confirmed that mutant p53 protein could lose its repressive function on UPase gene expression. These research achievements will benefit the management of clinical cancer patients.

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Appendices

1. Figures and tables
2. Reprint: Cao, D., Russell, R., Zhang, D., Leffert, J. J., and Pizzorno, G. Uridine phosphorylase (-/-) murine ES cells clarify the key role of this enzyme in the regulation of the pyrimidine salvage pathway and in the activation of fluoropyrimidines. Cancer Res. 62: 2313-2317, 2002
3. Award certificate: AACR-Aventis Scholar-in-Training Award

APPENDIX I: FIGURES AND TABLES

Fig. 1

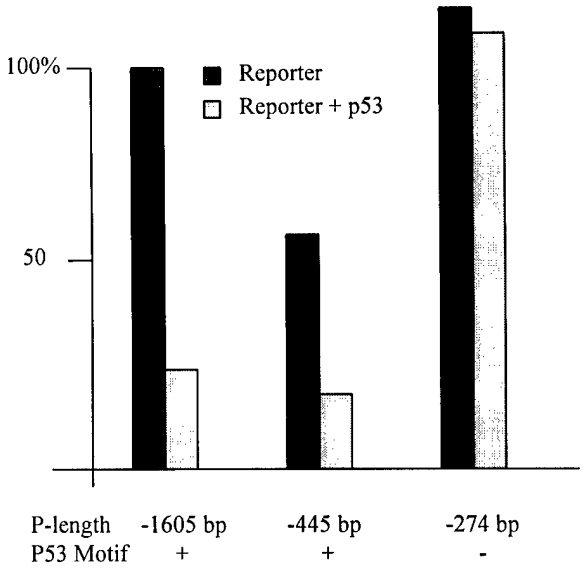

Fig. 2

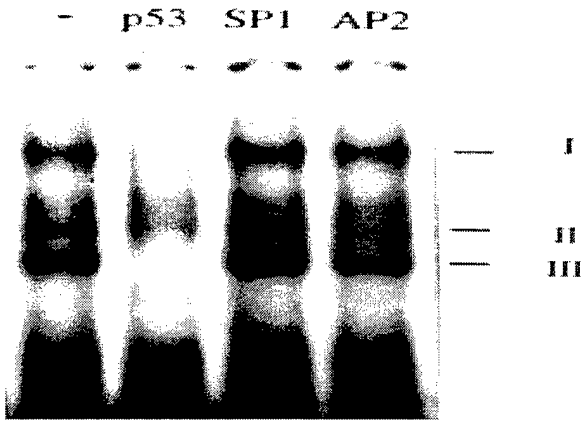

Figure 1 Promoter activity and wild-type p53 repression. The UPase promoter-Luciferase constructs -1605 bp (whole length), -445 (p53 motif +) and -274 (p53 motif -) display different promoter activity in EMT6 cells, and this p53 suppression action is confirmed by a Co-transfection with murine wild-type p53 gene.

Figure 2 Gel mobility shift assay. A radiolabeled double-stranded DNA probe (34-bp long) containing the p53 promoter-binding region was incubated with NIH 3T3 cell extract and separated on a 6% polyacrylamide gel. To determine binding specificity, cold p53 and other control probes were added as specific and nonspecific competitors, as indicated above the corresponding lanes.

Fig. 3

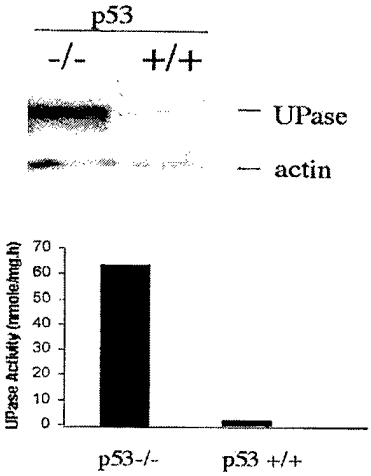

Fig. 4

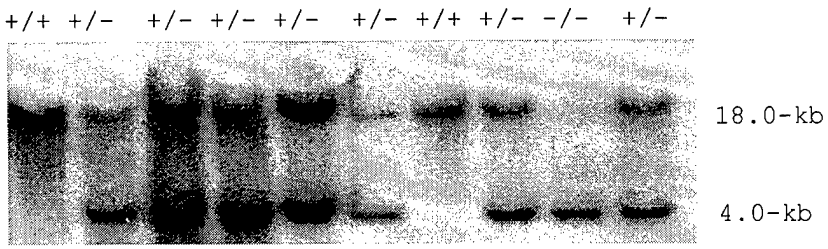

Figure 3 Analysis of UPase gene products in MEF p53 -/- and MEF p53 +/+ cells. Upper panel, Western blotting indicates UPase protein enrichment in MEF p53 -/- and the lower panel shows the difference in UPase activity.

Figure 4 Genotyping analysis of one litter of F2 mice from a cross between two UPase +/- mice. Tail genomic DNA was prepared and digested by *BamHI*. The blot was hybridized with the 0.6-kb genomic DNA probe. *Left*, molecular size marker.

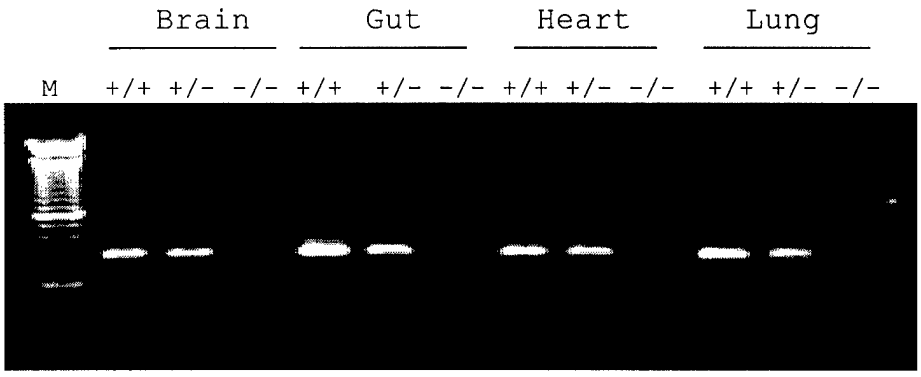

Figure 5 RT-PCR analysis of UPase gene mRNA isolated from organs from UPase +/+, UPase +/-, and UPase -/- mice. The forward primer (5'GCTCTTCCCGGATGAACACC) is located in exon 5, the targeted region, and the reverse primer (5'CGCCTGAAGTGCCAATGC) is in exon 6. There is no UPase mRNA present in the organs from UPase -/- mouse.

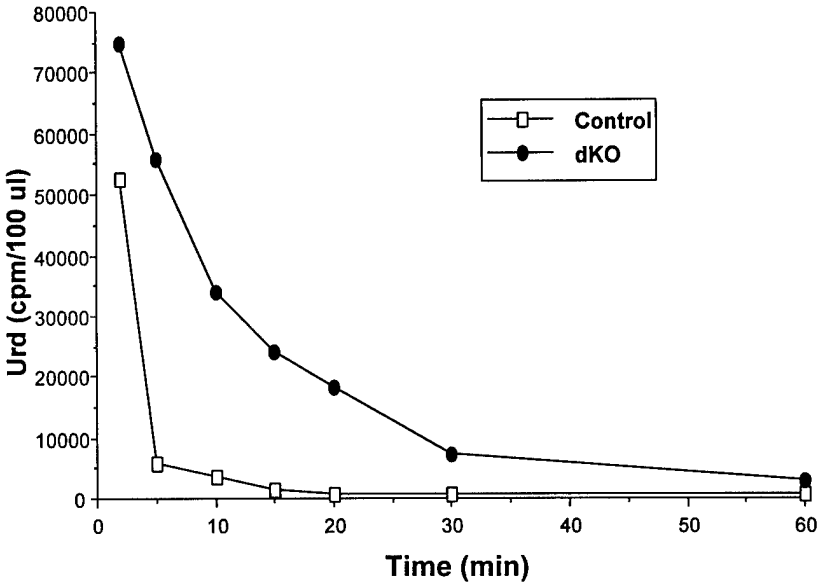

Figure 6 Clearance rate of [^{3}H] Uridine. A trace dose (25 μCi) of [^{3}H] uridine was administrated (i.p.) to UPase +/+ (control) and -/- (dko) and the presence of plasma [^{3}H] uridine was determined by HPLC analysis at time points as indicated. The half-life of [^{3}H] uridine in UPase -/- is much longer than that in UPase +/+.

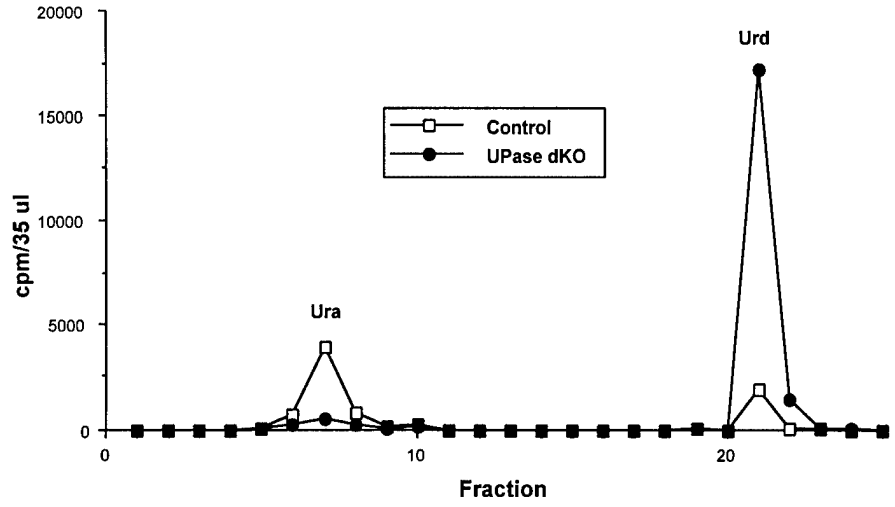

Figure 7 Metabolic fate of [^{3}H] Uridine. Blood samples were collected at minutes after [^{3}H] uridine administration by i.p. and analyzed by HPLC. An obvious uracil peak is shown in UPase +/+ mice, but not in UPase -/- ones.

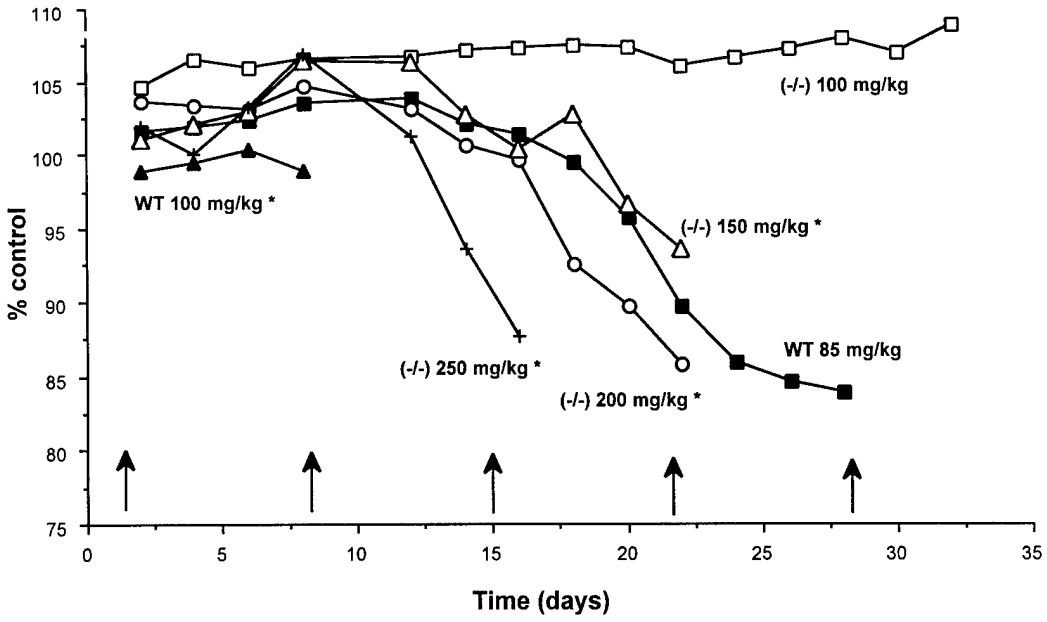

Figure 8 Response of UPase -/- mice to 5-FU administrated weekly. The drug was administered once a week i.p. (arrows) at the indicated doses on the lines. The mice were weighed every other day, and the number represents the average of 6 mice in each treatment group. The observation was terminated in each group after the first animal death according to University guidelines for the humane treatment of animals.

Table I Activity of enzymes in UPase -/- mice (nmol/mg/h)

	-/-				+/+			
	UPase	**TPase**	**UK**	**OPRTase**	**UPase**	**TPase**	**UK**	**OPRTase**
Brain	DN	ND	3.83	1.51	6.52	ND	3.90	1.48
Lung	ND	ND	2.78	ND	37.36	ND	2.72	ND
Heart	ND	ND	ND	ND	0.12	ND	ND	ND
Liver	ND	18.72	1.48	1.34	4.64	16.64	0.92	1.72
Spleen	ND	ND	7.24	ND	5.12	ND	5.56	ND
Gut	ND	3.84	ND	ND	576.80	4.24	ND	ND
Kidney	ND	ND	2.77	ND	32.63	ND	1.60	ND

Table II. Uridine Levels in Plasma, Urine, and tissues of UPase -/- Mice

	Plasma	Urine	Brain	Lung	Heart	Liver	Spleen	Gut	Kidney
UPase +/+	5.5	10.1	84.0	2.9	17.6	143.6	75.2	185.7	232.0
UPase +/-	12.3	42.1	ND	ND	ND	ND	ND	ND	ND
UPase -/-	32.3	240.4	193.4	58.2	213.7	181.3	235.0	390.2	340.4

[CANCER RESEARCH 62, 2313–2317, April 15, 2002]

Uridine Phosphorylase (−/−) Murine Embryonic Stem Cells Clarify the Key Role of this Enzyme in the Regulation of the Pyrimidine Salvage Pathway and in the Activation of Fluoropyrimidines[1]

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ABSTRACT

We have reported the elevation of uridine phosphorylase (UPase) in many solid tumors and the presence of a variant phosphorolytic activity in breast cancer tissues (M. Liu *et al.*, Cancer Res., *58:* 5418–5424, 1998). To better understand the biological and pharmacological significance of these findings, we have developed an UPase gene knockout embryonic stem (ES) cell model by specific gene targeting techniques. In this cellular model, we establish the critical role of UPase as an important anabolic enzyme in 5-fluorouracil (5-FU) activation and pyrimidine salvage pathway regulation. It has long been known that UPase regulates the plasma concentration of uridine; however, little is known of the role of UPase in the activation and metabolism of 5-FU and its derivatives, mainly because of the lack of an appropriate model system. The experimental data indicate that the disruption of UPase activity in murine ES cells leads to a 10-fold increase in 5-FU IC_{50} and a 2–3-fold reduction in its incorporation into nucleic acids, whereas no differences in toxicity is seen with other pyrimidine nucleoside analogues such as 5-fluorouridine, 2′-deoxy-5-fluorouridine, and 1-β-D-arabinofuranosylcytosine compared with WT (wild-type) ES cells. Benzylacyclouridine can specifically prevent the WT ES cells from the sensitivity of 5-FU. Our data also shows the effect of UPase on the cytotoxicity of 5′-deoxy-5-fluorouridine (5′DFUR), a 5-FU prodrug. The IC_{50} is increased almost 16-fold in the knockout cells compared with the wild type cells, demonstrating the role of UPase in catalyzing the conversion of 5′DFUR to 5-FU. These findings additionally elucidate the tumor-specific selectivity of capecitabine, the oral fluoropyrimidine prodrug approved for the treatment of metastatic breast and colorectal cancers.

Not only do the knockout cells present a decreased incorporation of 5-FU into nucleic acids but also an increased reliance on the pyrimidine salvage pathway. The reduced dependence of UPase knockout cells on the pyrimidine *de novo* synthesis is reflected in the apparent resistance to phosphonacetyl-L-aspartic acid, a specific inhibitor of pyrimidine pathway, with a 5-fold elevation in its IC_{50} in UPase-nullified cells compared with WT. In summary, we have successfully generated an UPase gene knockout cell model that presents reduced sensitivity to 5-FU, 5′DFUR, and phosphonacetyl-L-aspartic acid, although it does not affect the basic cellular physiology under normal tissue culture conditions. Considering the role of UPase in 5-FU metabolism and the elevated expression of this protein in cancer cells compared with paired normal tissues, additional investigation should be warranted to firmly establish the clinical role of UPase in the tumor selective activation of 5-FU and capecitabine.

INTRODUCTION

5-FU[3] still represents one of the most active antitumor agents in the treatment of solid tumors such as breast, colon, and head and neck cancers. Two main mechanisms of action contribute to the cytotoxic effect of 5-FU: (*a*) DNA-directed toxicity, where the formed 5-fluoro-dUMP tightly binds to thymidylate synthetase, resulting in inhibition of DNA synthesis and cell growth with a minor role played by DNA incorporation of the fluorodeoxynucleotides leading to the fragmentation of DNA and cell death; and (*b*) RNA-directed cytotoxicity with 5-FU incorporation into various RNA species, including polysomal RNA, nuclear RNA, and mRNA, thereby disrupting RNA maturation and functions (1–3).

Whereas the mechanisms of action have been well established, the contribution of the different pathways to 5-FU activation is still controversial because of the lack of an appropriate model system. 5-FU can be converted to 5-FUMP via the OPRTase pathway in the presence of phosphoribosyl PP_i or activated to 5-fluorouridine first and then to 5-FUMP via the UPase-kinase salvage pathway in the presence of R-1-P. 5-FU can also be converted to 5-fluorodeoxyuridine by TPase and then to 5-fluoro-dUMP by TK (1, 4). Some investigators have proposed that OPRTase plays a main role in the activation of 5-FU because of the limited pool of R-1-P available in the cells (5). However, Schwartz *et al.* (6) indicated that UPase is a critical enzyme in activation of 5-FU. This controversy arises from the presence of both UPase and OPRTase in the experimental models investigated thus far.

The clinical effectiveness of 5-FU is limited by its severe side effects such as myelosuppression, thrombocytopenia, and gastrointestinal lesions. Uridine has been used to reduce 5-FU toxicity leading to an increased therapeutic index (7). Several preclinical studies and clinical trials have demonstrated the ability of uridine to selectively protect normal tissues from 5-FU host toxicity. However, the clinical use of uridine rescue is hampered by its rapid clearance via degradation initiated by UPase in liver and dose-limiting toxicities resulting from high dose administration of uridine necessary to obtain the desired concentration for tissue protection. BAU, developed as an inhibitor of UPase, has been shown to be able to increase plasma uridine concentration by conserving endogenous uridine leading to similar protection of normal tissues (8–10). Finally, UPase was found to be elevated in most human tumors, and we have identified variant forms of UPase, particularly in breast tumors with various degrees of insensitivity to BAU providing the rationale for the increased selectivity of 5-FU-based therapy in these tumors (11). Currently, we have developed an UPase knockout ES cell model through gene targeting technology. Because of its clear genetic background, this model will provide a significant tool to elucidate the biological function of UPase and its role in 5-FU activation and uridine metabolism in cells. Here we report the effects of UPase disruption on pyrimidine metabolism and 5-FU antiproliferation.

MATERIALS AND METHODS

Cell Culture. Undifferentiated WT 129/JV ES murine cells and UPase knockout clones were maintained in gelatinized tissue culture flasks with high

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[3] The abbreviations used are: 5-FU, 5-fluorouracil; UPase, uridine phosphorylase; ES, embryonic stem; BAU, benzylacyclouridine; 5′DFUR, 5′-deoxy-5-fluorouridine; PALA, phosphonacetyl-L-aspartic acid; FUMP, fluorouridine monophosphate; OPRTase, orotate phosphoribosyl-transferase; R-1-P, ribose-1-phosphate; TPase, thymidine phosphorylase; MUP, murine UPase; NEO, neomycin resistance; TK, thymidine kinase; WT, wild-type.

glucose DMEM supplemented with 15% heat-inactivated fetal bovine serum, 2 mM glutamine, 0.1 mM β-mercaptoethanol, and 1000 units/ml of recombinant leukemia inhibitory factors (Life Technology, Inc., Grand Island, NY) at 37°C and 5% CO_2.

Construction of MUP Gene Targeting Vector and Selection of UPase Mutants. A 8.5-kb *Eco*RI-*Xho*I genomic DNA fragment containing the whole MUP gene (12) was subcloned into pBluescript KS II vector (Stratagene, La Jolla, CA). To disrupt UPase gene, we replaced with a 1.6-kb NEO gene (a positive selection marker) expression cassette a 2.5-kb fragment of UPase gene, which includes the 3′ part of intron 3, the whole exon 4 and intron 4, and the 5′ part of exon 5. The MUP/NEO fragment was subsequently inserted into a vector containing herpes simplex virus TK gene cassette, a negative selection marker, to exclude the nonrecombination (nontargeted) mutants, generating the targeting construct (Fig. 1).

The linearized MUP/NEO/TK Bluescript targeting construct was introduced by electroporation into 129/JV ES cells and the clones doubly selected by G418 and ganciclovir for the presence of NEO gene and absence of TK gene. The ES clones carrying the mutant UPase gene were identified by PCR and Southern blot. Double knockout cells (two alleles disrupted) were generated by exposing the clones to high concentrations of G418 up to 5.5 mg/ml (13).

Western Blot Analysis. ES cells were solubilized in 2× SDS gel loading buffer [50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% bromophenol blue, and 10% (v/v) glycerol], and the lysate was separated on a 15% SDS-PAGE and then transferred to polyvinylidene difluoride membrane (Hybond P; Amersham). UPase was detected using a polyclonal anti-UPase antibody generated in our laboratory diluted 1:100 in casein buffer [5% casein, 0.05% Triton X-100, 0.3 M NaCl, 50 mM citric acid, 0.3 M Tris base (pH 7.6)]. The membrane was reprobed by commercial antiactin monoclonal antibody to evaluate the amount of the protein loaded.

Cell Growth and Drug Sensitivity Assay. Cell growth rate was measured using a Cell Proliferation kit (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt; Boehringer Mannheim, Indianapolis, IN) in 96-well plates and absorbance at 500 nm, indicating the viable cell number, was determined with a Titertek Multiskan MCC340 (Huntsville, AL) microplate reader, using A^{750} as an internal control. The drug sensitivity assays were performed similarly as above. Next, 3000 cells/well were plated in gelatinized 96-well tissue culture plates. After overnight incubation, cells were treated with different drugs and harvested at indicated time points. Each concentration point was replicated in six wells, and all of the experiments were repeated at least twice.

Enzyme Activity Assays. UPase activity was measured by uridine conversion to uracil using a Tris-HCl lysate (11) incubated with 200 μM [^{3}H]uridine and 1 mM potassium phosphate. The UPase product, [^{3}H]uracil, was separated on silica TLC plates (Kieselgel 60; Merck) using an 85:15:5 mixture of chloroform, methanol, and acetic acid, respectively. Protein amounts were determined with protein assay dye (Bio-Rad Laboratories, Hercules, CA). TPase activity was similarly analyzed using 200 μM [^{3}H]thymidine as a substrate (11).

OPRTase activity was assayed by measuring the conversion of [^{14}C]fluorouracil into FUMP in the presence of 200 μM [^{14}C]fluorouracil, 1 mM phosphoribosyl PP_I, and 100 μM $MgCl_2$ (3).

Uridine kinase was determined by the conversion of [^{3}H]uridine into [^{3}H]UMP with 200 μM [^{3}H]uridine, 1 mM ATP, and 100 μM $MgCl_2$ (3).

R-1-P concentration was measured in the presence of 50 μM [^{14}C]fluorouracil and 5 μg pure recombinant UPase protein followed by TLC separation as indicated above (11).

Incorporation of [^{3}H]5-FU and [^{3}H]Uridine into Nucleic Acids and Measurement of Ribonucleotide Triphosphate Pools. Cells (5×10^5/flask) were incubated for 24 h in medium containing [^{3}H]5-FU (5 μM) or [^{3}H]uridine (2 μM), washed twice with cold PBS, and the macromolecular precipitated with 15% trichloroacetic acid. The final precipitates were dissolved in tissue solubilizer (TS-1) and the radioactivity determined.

The cell supernatants obtained with the TCA precipitation were neutralized 1 N trioctylamine in freon. The nucleoside triphosphates were eluted isocratically on an high-performance liquid chromatography anion exchange column (Partisil-10-SAX; Altex) using 0.4 M $NH_4H_2PO_4$ (pH 4.5) as mobile phase.

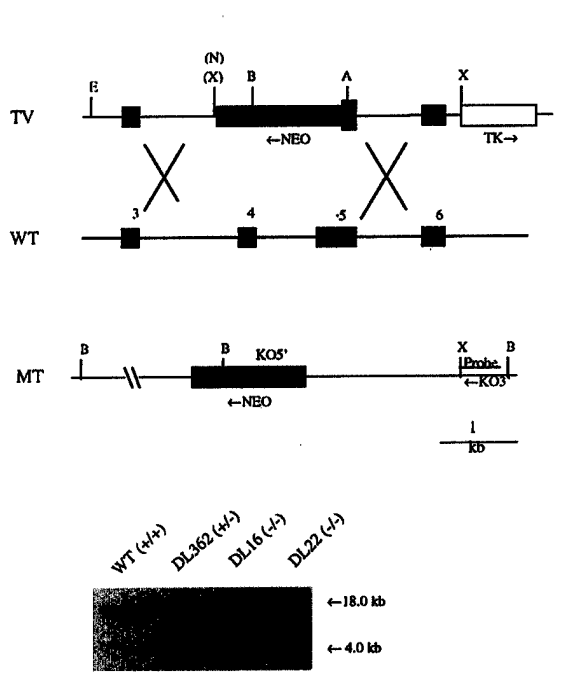
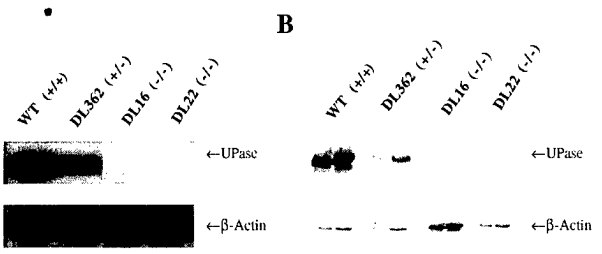


Fig. 1. Targeted disruption of UPase locus. *A*, partial restriction map of UPase locus in genome, targeting vector, and the targeted allele. A 2.5-kb genomic fragment of MUP (*Nhe*I-*Apa*I) was replaced by a 1.6-kb *NEO* cassette. *TK* gene cassette was flanked at 3′ end. *Arrows* indicate the orientations. *TV*, targeting vector; *WT*, wild type allele; and *MT*, mutant allele. *A*, *Apa*I; *B*, *Bam*HI; *E*, *Eco*RI; *(N)*, *Nhe*I and *(X)*, *Xba*I, both were blunted; and *X*, *Xho*I. KO5′ (5′CGGCTTTATACATGGCGTAGCG) and *KO3′* (5′GTGATGGTTTTCAAGGTCCTTTGC) are primers for PCR screening of mutants. *B*, Southern blot analysis of the UPase gene locus. The genomic DNA was digested by *Bam*HI, and the blot was hybridized with the 600-bp PCR fragment immediately outside of *Xho*I cloning site. The WT band is ~18 kb, and the length of the disrupted allele is 4 kb, because of the introduction of a *Bam*HI site in NEO cassette. *Left*, molecular size markers. *Lane 1*, WT; *Lane 2* single knockout clone; and *Lanes 3* and *4*, two double knockout clones.

RESULTS

Targeted Disruption of the MUP Gene and Abrogation of UPase Expression in ES Cells. To disrupt the MUP gene and abrogate the expression of its functional product, we replaced a 2.5-kb fragment of the UPase gene, which contains the 3′ part of intron 3, the whole exon 4 and intron 4, and the 5′ part of exon 5, with a 1.6-kb NEO gene cassette, leading to a 92 and 1/3 amino acid deletion, and a shifting mutation of downstream codons (out of frame). After a TK gene expression cassette was flanked at the 3′ end of the UPase gene fragment, the linearized targeting construct was electroporated into murine 129/JV ES cells. The transfected cells were then exposed to G418 and ganciclovir to select for the targeted UPase mutant clones. Analysis of 38 survival clones by PCR and Southern blot hybridization indicated that 13 of them underwent a correct homologous recombination, resulting in the targeted UPase gene disruption (data not shown). Clone DL362 containing the single allele UPase mutation was expanded and exposed at 5.5 mg/ml G418 for 2 weeks to select for UPase double allele knockout clones (13). The resultant 50 clones were subjected to Southern blot analysis, and 5 of them were found to have the targeted disruption at both alleles (Fig. 1).

The expression products of the disrupted UPase gene were evaluated in two double knockout clones, DL16 and 22, and their parental single knockout cell clone DL362. A WT parental 129/JV ES clone was also used as a control. A 600-bp UPase cDNA probe, corresponding to exons 3–7, identified a 1.4-kb UPase RNA in WT and single knockout cells. This RNA species was not detectable in the two double knockout clones DL16 and 22. The abundance of UPase mRNA in single knockout cells was ~50% of the WT control cells indicating that the UPase gene disruption results in the reduction of



C

Cells	UPase Activity (nmoles/mg/min)
WT (+/+)	101.45±5.23
DL362 (+/-)	60.70±4.36
DL16 (-/-)	2.12±1.68
DL22 (-/-)	1.98±1.08

Fig. 2. UPase gene expression in the knockout clones. *A*, Northern blot analysis of UPase gene transcripts. Total RNA (10 μg) was used in each lane, and the blot was sequentially probed with mouse UPase and β-actin cDNA random-labeled with ^{32}P. A 1.4-kb RNA band is seen in WT and single knockout (halved in density) cells but not detectable in double knockout cells. *Lane 1*, WT; *Lane 2*, single knockout clone; and *Lanes 3* and *4*, two double knockout clones. *B*, Western blot analysis of UPase protein. Total cell lysates were separated on 15% SDS-PAGE, and the protein blot was hybridized sequentially with UPase polyclonal and β-actin monoclonal antibodies. A M_r 36,000 protein band is seen in WT and single knockout cell lysates but not in the double knockout cell lysate. *Lanes 1* and *2*, WT; *Lanes 3* and *4* single knockout clone; *Lanes 5*, and *6*, double knockout clone DL16; and *Lanes 7* and *8*, double knockout clone DL22. *C*, UPase activity assays. The cell lysates from WT and knockout cell clones were used to check the ability to convert [^{3}H]uridine to [^{3}H]uracil. The activity is expressed in nmol/mg protein/min.

the mRNA transcripts (Fig. 2). To evaluate whether any translation compensation occurs in single knockout cells and exclude the possibility that the truncated and shifted UPase protein was still expressed in the double allele knockout cells, Western blot analysis was performed using an anti-UPase polyclonal antibody generated in our laboratory (11). The data indicate that the expression of the UPase protein was abrogated in the two double knockout clones and halved in the single knockout clone DL362 (Fig. 2). UPase activity was also assayed in these cell extracts indicating that no activity was present in the two double knockout clones, and the uridine conversion was reduced to 50% in single knockout cells compared with the WT control cells (Fig. 2).

To analyze the effects of UPase gene disruption on cellular physiological function, the double knockout clones DL16 and 22, the single knockout clone DL362, and a WT clone were cultured and assayed in regular medium to determine any change in their proliferative rate. We did not observe any obvious difference in growth rate between WT and knockout cells (data not shown). Moreover, the sizes of both pyrimidine and purine ribonucleotide pools did not change, and the Na^+-dependent active transport of uridine was not affected (data not shown). More interestingly, the intracellular level of R-1-P, a cosubstrate in the phosphorolytic reaction, was not significantly altered in the knockout clones with a concentration of 2 ± 0.15 nmol/mg of proteins in the WT cells and 2.23 ± 0.19 nmol/mg of proteins in the double knockout cells.

UPase Knockout Cells: Effect on Sensitivity to Pyrimidine Analogues. To elucidate the effects of the disruption of UPase activity on cell drug sensitivity, double knockout clones DL16 and 22, the single knockout clone DL362, and the WT ES clone were tested against five pyrimidine analogues and a DNA intercalating antitumor agent, doxorubicin. A 72-h exposure to 5-FU indicated a reduced sensitivity to this pyrimidine antimetabolite with a 10-fold increase in IC_{50} from 0.2 μM for the WT cells to 2.0 μM for the two UPase double knockout clones (Fig. 3). The single knockout cells still maintained sensitivity to 5-FU with an IC_{50} of 0.35 μM. This difference in sensitivity is reflected in the 5-FU incorporation into the nucleic acids of these double knockout cells with a reduction of 2–3-fold compared with the WT cells (Table 1). When the cells were exposed simultaneously to 5-FU in the presence of the specific UPase inhibitor BAU (50 μM), we observed a reduction in 5-FU activity in both WT and UPase single knockout ES cells with IC_{50}s similar to the ones determined for the double knockout clones. The main drawback of the antineoplastic activity of 5-FU is its toxic effect against normal tissues, mostly gastrointestinal mucosa and hematopoietic system. One of the strategies to reduce the toxic side effects of 5-FU has been to administer a nontoxic prodrug that can be selectively activated at tumor level. 5′DFUR represents one of these examples (5, 14). Our results indicated that WT and UPase single knockout cells were much more sensitive to 5′DFUR than the double knockout cells, with IC_{50} of 0.5, 2.5, and 8.0 μM for the WT, single knockout, and double knockout cells, respectively (Fig. 4), establishing the importance of UPase in the activation of 5′DFUR. The abrogation of UPase had no effects on the cytotoxicity of 2′-deoxy-5-fluorouridine, mainly activated by TK, and 1-β-D-arabinofuranosylcytosine, a deoxycytidine analogue. UPase activity also did not affect the cytotoxic activity of a DNA intercalator and topoisomerase inhibitor such as doxorubicin (data not shown).

Role of UPase Activity on Pyrimidine Salvage Pathway. PALA, a transitional state analogue, intermediate in the condensation of carbamylphosphate with L-aspartic acid, can efficiently inhibit the pyrimidine *de novo* synthesis and deplete the pyrimidine nucleotide

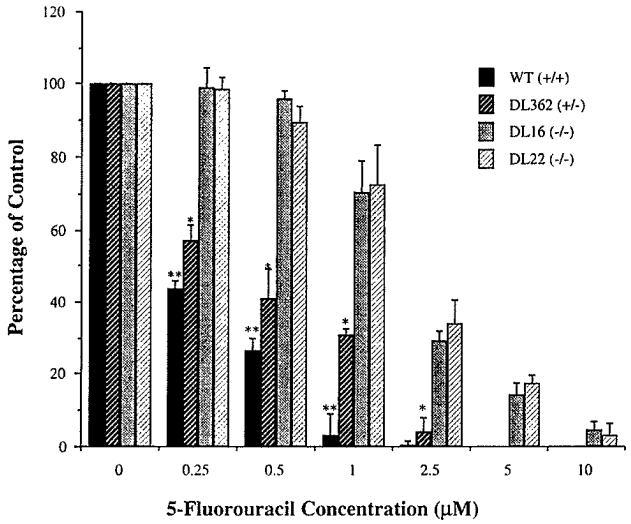


Fig. 3. Antiproliferative activity of 5-FU in WT and knockout ES cells. The WT and knockout ES cells were exposed to different concentration of 5-FU for 72 h, and the amount of viable cells are determined by cell proliferation kit (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt). Each column represents the mean of three experiments; *bars*, ± SD. *, significantly different from the double knockout cells given the same treatment ($P < 0.001$, unpaired *t* test), **, significantly different from both single ($P < 0.05$) and double ($P < 0.001$) knockout cells (unpaired *t* test).

Table 1 *Incorporation of radio-labeled 5-FU and uridine*

Cell line	[^{3}H]5-FU (pmol/10^6 cells/24 h)	[^{3}H]Uridine (nmol/10^6 cells/24 h)
WT (+/+)	56.67 ± 5.69	2.48 ± 0.23
DL362 (+/−)	46.88 ± 8.17	2.73 ± 0.35
DL16 (−/−)	21.18 ± 3.31	3.29 ± 0.39
DL22 (−/−)	16.88 ± 5.67	3.39 ± 0.19

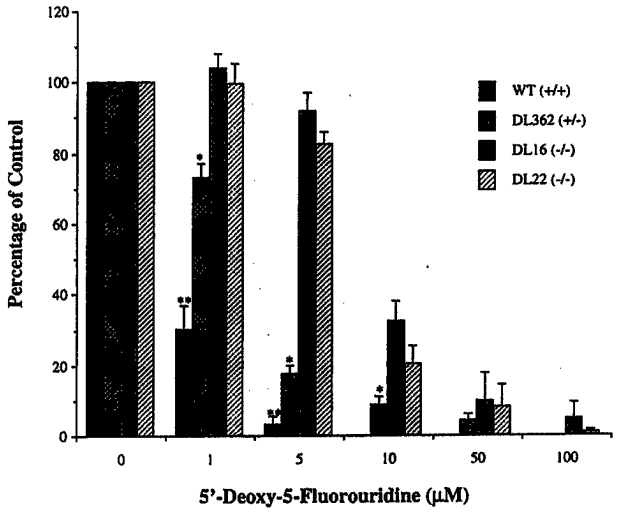


Fig. 4. Role of UPase in the activation of 5'DFUR. WT and knockout ES cells were exposed to different concentration of 5'DFUR for 72 h, and the cell were treated as described in Fig. 4. Each column represents the mean of three separate experiments; *bars*, ± SD. *, significantly different from the double knockout cells given the same treatment ($P < 0.01$, unpaired t test); **, significantly different from both single ($P < 0.01$) and double ($P < 0.001$) knockout cells (unpaired t test).

pools via inhibition of aspartate transcarbamylase (15). Our data demonstrate that the disruption of UPase activity causes an increase in the IC_{50} of PALA from 50 μM in WT ES cells to >2000 μM for the double knockout cells (Fig. 5), indicating the diminished role of the *de novo* pyrimidine synthesis in this knockout cell model. This is confirmed by the increased uridine incorporation (Table 1) and uridine kinase activity (Table 2). As expected, uridine rescue (50 μM) could efficiently protect both WT and single knockout cells from PALA toxicity (data not shown). The elevated uridine kinase activity observed in the UPase double knockout cells is reflected in an increased sensitivity of these murine ES subpopulations to 5-fluorouridine, a direct substrate for uridine kinase. The 0.05 μM ED_{50} of 5-fluorouridine in WT ES cells was reduced to 0.02 μM in both UPase double knockout clones. No change in sensitivity to 5-fluorouridine was observed in the UPase single knockout ES cells that did not display any significant alteration in uridine kinase activity. The analysis of TPase, an enzyme that despite a lower efficiency shares substrate specificity with UPase, surprisingly revealed that no detectable activity was present in WT ES cells, and no induction was observed in the UPase knockout ES cells. Similarly, eliminating UPase activity did not cause any alteration in the expression of OPRTase, a key enzyme in the *de novo* biosynthetic pathway and in the activation of 5-FU (Table 2).

DISCUSSION

Using specific gene targeting methodology, we were successfully able to obtain several UPase knockout ES clones. Northern blot and Western blot analyses as well as enzyme activity assays confirmed the absence of the UPase gene products in the double knockout cells and an ~50% reduction in the single knockout cells. These cell clones together with their parental WT cells compose an ideal cell panel, genetically differing only in UPase activity alone, to evaluate the metabolism and the activity of pyrimidine and antipyrimidine metabolites.

Theoretically, the abrogation of UPase activity, the first enzyme of the pyrimidine degradative pathway, should lead to the accumulation of uridine and consequent expansion of pyrimidine nucleotide pools in knockout cells. However, we did not find significant changes in ribonucleotide pools, in the intracellular level of R-1-P, and in the activity of the enzymes involved in pyrimidine regulation except for uridine kinase that was found elevated in knockout cells.

Uridine homeostasis is apparently maintained by a reduced contribution of the pyrimidine *de novo* synthesis as indicated by a reduced sensitivity of the double knockout cells to PALA and by a greater reliance on the pyrimidine salvage pathway as confirmed by an increased incorporation of preformed pyrimidines (uridine) in the nucleic acids.

Many investigators have reported that UPase is mainly a catabolic enzyme leading to the formation of β-alanine by catalyzing uridine phosphorolysis rather than the ribosylation of uracil (5, 16, 17). Unlike purine bases that can be salvaged by adenine phosphoribosyltransferase and hypoxantine-guanine phosphoribosyltransferase in a single step reaction, pyrimidine salvage is thought to occur only at the nucleoside level (16). However, recent observations have indicated that UPase may function as an anabolic enzyme using activated ribose to salvage uracil into uridine nucleotides (with uridine as an intermediate) even in the presence of excess inorganic phosphate (6, 18, 19).

Although two main pathways contribute to 5-FU activation, either via the OPRTase pathway in the presence of phosphoribosyl PP, or via UPase-initiated salvage pathway with R-1-P as cosubstrate, it is still controversial as to which pathway plays a predominant role. Our results in UPase knockout ES cells support the hypothesis that UPase substantially contributes to the activation of 5-FU as indicated by a 10-fold increase in IC_{50} for 5-FU, and a reduced 5-FU sensitivity in WT and single knockout cells in the presence of the UPase inhibitor BAU.

We have indicated the role of R-1-P as the rate-limiting factor (5, 20) *in vivo* of the anabolic reactions catalyzed by UPase, and con-

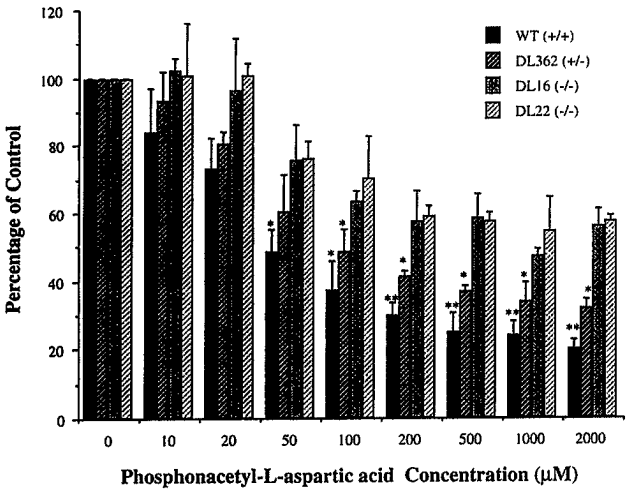


Fig. 5. Disruption of UPase activity results in resistance to PALA. WT and knockout ES cells were exposed to different concentrations of PALA for 72 h, and the cells were treated as described in Fig. 4. Each column represents the mean of three separate experiments; *bars*, ± SD. *, significantly different from the double knockout cells given the same treatment ($P < 0.05$, unpaired t test); **, significantly different from both single ($P < 0.01$) and double ($P < 0.005$) knockout cells (unpaired t test).

Table 2 *Activities of uridine kinase and OPRTase*

Cell line	Uridine kinase (nmol/mg protein/h)	OPRTase (nmol/mg protein/h)
WT (+/+)	185.9 ± 12.3	103.3 ± 11.5
DL362 (+/−)	188.2 ± 15.9	102.5 ± 10.8
DL16 (−/−)	390.6 ± 11.8	119.5 ± 12.5
DL22 (−/−)	322.4 ± 13.1	107.8 ± 13.5